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Mutations in PFCRT K76T do not correlate with sulfadoxine–pyrimethamine–amodiaquine failure in Pikine, Senegal

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Abstract

In 2003, the high level of chloroquine (CQ) treatment failure for uncomplicated *Plasmodium falciparum* malaria cases has led Senegal to adopt a new combination therapy with sulfadoxine–pyrimethamine and amodiaquine (SP-AQ). From September through November 2004, we used the 14-day World Health Organization follow-up protocol to assess the therapeutic response in patients with uncomplicated *P. falciparum* malaria in an area of high prevalence of *pfprt* T76 mutant allele and SP resistance mutations. Of the 82 patients who were recruited, 68 (82.9%) completed follow-up. The response of the patients to treatment was adequate clinical response for 63 out of 68 patients (92.6%), while five (7.4%) clinical failures were recorded, four early treatment failures, and one late treatment failure. The prevalence of the *pfprt* T76 allele at day 0 was 59.5%. The two-sided Fisher's exact test did not show an association between *pfprt* T76 allele and treatment failure ($p=0.167$). The transitory treatment is effective and safe. However, the presence of high levels of mutant alleles points out the need to closely monitor the new therapeutic regimen.

Introduction

Resistance of *Plasmodium falciparum* to cheap antimalarials remains a threat for sub-Saharan African countries. High rates of chloroquine (CQ) treatment failures (Gaye et al. 1999) and up to 11-fold increased risk of childhood malaria death (Trape et al. 1998) prompted Senegalese health authorities to abandon chloroquine in 2003 for the treatment of *P. falciparum* malaria. To further counter the threat of resistance of *P. falciparum* to monotherapies and to improve treatment outcome, combinations of antimalarials are now recommended by the World Health Organization (WHO) for the treatment of falciparum malaria (WHO 2006). During a consensus meeting held in 2003, Senegalese health authorities adopted an interim option using sulfadoxine–pyrimethamine (SP) plus amodiaquine (AQ), known as SP-AQ, for falciparum malaria, despite the fact that SP and AQ failures have been documented in the country when used alone (Botella et al. 1991; Brasseur et al. 1999; Gaye et al. 1999; Sokhna et al. 2001).

The new combination therapy (SP-AQ) is expected to suppress or at least delay the surge of *P. falciparum* malaria drug resistance, as both molecules have independent modes of action and unrelated biochemical targets. AQ, a 4-aminoquinoline, has a mechanism of action very similar to that of CQ (Brasseur et al. 1999). Despite cross-resistance with CQ, AQ may be effective in areas with high-level resistance to CQ (Graupner et al. 2005). Evidence for the association of AQ and its metabolites and CQ cross-resistance with specific changes on the *pfcr* gene at positions 72–76 has been postulated as a predictor of AQ's clinical efficacy in vivo (Warhurst 2003; Ochong et al. 2003).

As drug resistance has been linked to mutations in specific loci (i.e., *pfcr* has been linked to chloroquine resistance and *dhps–dhfr* to sulfadoxine–pyrimethamine), our study investigated the treatment outcome in an area with high-level *pfcr* K76T associated mutations (Thomas et al. 2002; Bertin et al. 2005; Sarr et al. 2005).

Materials and methods

Study site and population and collection of blood samples

The study took place from September to November 2004, and 82 patients were recruited in Pikine, where malaria transmission is seasonal (Robert et al. 2000). Patients aged 3 years or higher with axillary temperature $\geq 37.5^{\circ}\text{C}$ or recent history of fever and infected with *P. falciparum* as determined by blood/thick smear were enrolled.

Patients were excluded if they presented with symptoms of severe or complicated malaria, if another *Plasmodium* species in addition to *P. falciparum* was detected in their blood, if they manifested clinical symptoms of chronic disease such as tuberculosis, HIV, or chronic diarrhea. A history of allergic reaction to sulfonamides or a potential problem with follow-up, i.e., a trip outside the Pikine area was also part of the exclusion criteria. Pregnancy was not an exclusion criterion.

Informed consent was obtained from patients or in the case of children, from parents or guardians. Ten milliliters of peripheral blood was collected into EDTA tubes upon enrolment, and blood was applied to filter paper, dried, and stored in plastic bags with silica gel at ambient temperature. The institutional review board at the Harvard School of Public Health and the Senegalese Ministry of Health approved the study.

Parasite density assay

Thick and thin blood films were stained with Giemsa. They were observed immediately by light microscopy by an experienced laboratory technician and re-read in our laboratory at the

hospital. The number of parasites per microliter of blood was calculated by comparing the number of infected red blood cells per 200 leukocytes with the average white blood cell count of 8,000 per microliter. Then, parasite density was calculated by using the following formula: parasitemia per microliter=number of infected red blood cells $\times$ 8,000/200 leukocytes.

Treatment

The patients received a single oral dose of SP (25 mg per kg body weight of sulfadoxine plus 1.25 mg per kg body weight of pyrimethamine) and oral AQ (total dose of 30 mg per kg body weight in three equally divided daily doses). All doses were administered under medical supervision and patients were directly observed for 30 min, allowing the medical team to monitor acute adverse drug reactions such as vomiting. Those who vomited while under observation received the same repeat doses. Patients were followed by home visits on days 3, 7, and 14. Clinical and parasitologic responses were evaluated on the aforementioned days and any other day in case of aggravation.

DNA extraction and amplification

We used for genotyping DNA obtained either from filter paper or from whole blood. DNA was extracted from filter paper using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA from whole blood was extracted using the phenol chloroform isoamyl alcohol method as described by Viriyakosol et al. (1995). Briefly, after lysis of the red blood cells in 0.05% saponin, the parasites were treated with proteinase K, and then the DNA was extracted by phenol chloroform isoamyl alcohol, precipitated with ethanol, and resuspended in TE buffer. The region surrounding the codon 76 for the *pfcr* gene was amplified by PCR followed by restriction fragment length polymorphism analysis to distinguish wild-type allele from mutant allele (Djimde et al. 2001a). The three sequence families of the merozoite surface protein-1 block 2, *Msp1* (K1, Mad20, Ro33) were typed to distinguish between monoclonal and multiclonal infections.

Assessment of treatment efficacy

The study team used the WHO criteria to classify responses to treatment (WHO 1996). Treatment efficacy is classified as early treatment failure (ETF) if the patient develops danger signs or severe malaria on days 1, 2, and 3 in the presence of parasitemia; or parasitemia on day 2 > day 0 count, with axillary temperature $\geq 37.5^{\circ}\text{C}$; or parasitemia exists on day 3 with axillary temperature $\geq 37.5^{\circ}\text{C}$ or parasitemia on day 3 is $25\% \geq$ count on day 0.

The response is classified as late treatment failure (LTF) if the patient develops danger signs or severe malaria after day 3 in the presence of parasitemia, without previously meeting any of the criteria of ETF, or parasitemia and axillary temperature $\geq 37.5^{\circ}\text{C}$ (or history of fever) on any day from day 4 to day 14, without previously meeting any of the criteria of ETF.

The response to treatment will be classified as adequate clinical response (ACR) if the patient shows one of the following conditions during the follow-up period (up to day 14): absence of parasitemia on day 14 irrespective of axillary temperature, without previously meeting any of the criteria of ETF or LTF or axillary temperature $< 37.5^{\circ}\text{C}$ irrespective of the presence of parasitemia without previously meeting any of the criteria of early or late treatment failure.

Patients who failed to respond to treatment were given quinine 10 mg/kg (IM) two times a day for 3 days.

Statistical analysis

Baseline frequencies of mutations were determined on samples taken before treatment. Mixed infections were considered as mutant type for analysis convenience. The two-sample *t* test was

used to compare two sample means. A p value <0.05 was considered as being statistically significant. The analysis were done using Stata version 9.0 (StataCorp, College Station, TX, USA).

Results

From September to November 2004, a total of 82 patients with uncomplicated *P. falciparum* malaria were recruited, 68 completed the 14-day follow-up while 14 were lost to follow-up or excluded. Patients who completed the follow-up did not present any differences in age, parasite density, and temperature with those lost to follow-up, as shown in Table 1. The in vivo and molecular analyses included only those who completed the follow-up.

Analysis of in vivo data

In total 63 patients out of 68 (92.6%) have presented an ACR to the treatment, while five (7.4%) clinical failures were recorded, four ETF, and one LTF. All ETF were defined as the presence of parasites on day 3 with axillary temperature $\geq 37.5^{\circ}\text{C}$. The LTF was determined for a patient with the presence of parasites and axillary temperature $\geq 37.5^{\circ}\text{C}$ after day 3. For patients who have completed the follow-up, we did not record any severe side effect.

Analysis of molecular data

The prevalence of *pfprt* T76 allele at day 0 was 59.5%. Of the five individuals who failed, three harbored at day 0 the wild-type allele K76 while two possessed the mutant allele T76. At baseline, the two-sided Fisher's exact test did not show an association between *pfprt* T76 allele and treatment failure ($p=0.167$). At day 0, the *msp-1* typing has shown that 36.14% of the samples that yielded PCR product were multiclonal infections. *Pfprt* and *msp-1* typing were not done in the case of failure.

Discussion

The present study investigates SP-AQ efficacy in an area with known high prevalence of *pfprt* K76T polymorphism (Thomas et al. 2002; Bertin et al. 2005; Sarr et al. 2005) and SP resistance mutations (Ndiaye et al. 2005). However, the prevalence of *pfprt* T76 mutant allele has decreased significantly in 1 year, from 2003 (Sarr et al. unpublished data) the last year of chloroquine use, to 2004, the first year of SP-AQ use ($p=0.0495$). This finding supports previous findings in Malawi, where the withdrawal of chloroquine was accompanied by a diminution of the prevalence of *pfprt* T76 allele from 17% to 2% between 1998 and 2000 (Mita et al. 2003). Another study in the same country showed the same trend with the prevalence of *pfprt* T76 allele at 85% in 1992 and 13% in 2000 in the capital city (Kublin et al. 2003). Theoretically, this situation is explained by the absence of chloroquine pressure allowing the wild-type K76 allele to re-emerge. A less rapid decrease of the prevalence of the *pfprt* T76 allele was seen in China, where not all chloroquine pressure against *P. falciparum* would have been removed (Wang et al. 2005). We believe that the prevalence of the *pfprt* T76 allele may rapidly diminish after the end of chloroquine use.

In Pikine, the combination of SP-AQ was safe. We did not record any severe SP-AQ-related adverse events during the follow-up as noticed in other areas (Brasseur et al. 1999; Zongo et al. 2005; Menard et al. 2005) even if AQ used in prophylaxis has been involved in agranulocytosis, hepatotoxicity, and anemia (Olliaro and Mussano 2002). However, we did not investigate biologic parameters such as hemoglobin levels or hepatic functions to check the aforementioned abnormalities.

The treatment was also effective with 92.6% of adequate clinical response even if the treatment efficacy is lower than what has been described in other settings in Senegal (Faye et al. 2007). However, SP (Sokhna et al. 2001) and AQ (Brasseur et al. 1999) failures have been noticed in other regions of Senegal when used alone, suggesting close monitoring of treatment outcomes because of the presence of SNPs in *pfcr* and *dhfr-dhps* genes. We investigated the relationship between *pfcr* T76 mutation in Pikine and clinical outcome, knowing that this is the first year of the combination therapy since several decades of chloroquine use. The two-sided Fisher's exact did not show any association ($p=0.1667$) between the mutant allele and the treatment outcome. There is a significant difference ($p<0.001$) between parasite carriers by day 14 in 2001 (Sarr et al. unpublished data) when patients were treated with CQ and 2004 when they received the SP-AQ.

The two-sided Fisher's exact did not show any significant difference in terms of age between those who were treated successfully and those who failed ($p=0.87$). Treatment failure was not related to age.

The local National Malaria Control Program has considered the SP-AQ regimen as an interim step, but one could ask if there was a need to switch to ACT when the SP-AQ treatment was affordable, safe, and effective as shown in Cameroon (Basco et al. 2002). We believe that the SP-AQ drug regimen needs to be closely monitored because of the presence of *pfcr* and *dhfr* and *dhps* mutant alleles and since the other alternative drugs are more expensive. The direct cost of an adult treatment with SP-AQ is less than one-third than that of an adult course at co-administered artemisinin plus amodiaquine (MacIntosh 2000).

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randomized controlled trial from Burkina Faso. Am J Trop Med Hyg 2005;73:826–832. [PubMed: 16282288]

Table 1

Demographic and baseline characteristics of study patients who completed the follow-up and those who were excluded, in 2004

Variables	Complete follow-up	Exclusions	<i>p</i> value
Mean age, years	19.7 (8)	19.1 (12)	0.83
Geometric mean parasite density D0 (parasite/ μ l)	37,861 (30,198)	51,318 (45,064)	0.32
Mean temperature D0 ($^{\circ}$ C)	39 (0.9)	38.88 (0.76)	0.59

The data show the means of variables with their standard deviations in brackets.

D0 Day of inclusion